Regulatory Toxicology and Pharmacology 56 (2010) 290–299

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

The safety of chitosan as a pharmaceutical excipient

Paul Baldrick

Scientific and Regulatory Consulting, Covance Laboratories Ltd., Otley Road, Harrogate, North Yorkshire HG3 1PY, UK

ARTICLE INFO

Article history:
Received 5 August 2009
Available online 27 September 2009

Keywords:
Chitosan
Excipient
Safety evaluation
Biological activity
Pharmacokinetics
Toxicology

ABSTRACT

Interest in use of the polysaccharide chitosan as a pharmaceutical excipient by different dose routes and for a number of applications is not new but it still does not appear to be present in any marketed drugs. Including a novel excipient in a new drug formulation requires a number of safety considerations. Review of the published literature showed that chitosan has low oral toxicity and local tolerance potential supporting use in non-parenteral formulations. Prior human oral exposure has occurred through use of chitosan dietary supplements and food additive, medical device and cosmetic applications. Although systemic exposure to parent chitosan may be limited (due to digestion in the gastrointestinal tract), any that is absorbed will likely undergo enzyme degradation to naturally occurring glucosamine, and *N*-acetylglucosamine, its copolymers, which are excreted or used in the amino sugar pool. Chitosan has local biological activity in the form of haemostatic action and, together with its ability to activate macrophages and cause cytokine stimulation (which has resulted in interest in medical device and wound healing applications), may result in a more careful assessment of its safety as a parenteral excipient.

1. Introduction

Excipients are vital components of drug formulations with a range of materials having an established clinical use for many years. However, development of new excipients has been slow, a fact partly related to cost but also regulatory hurdles to demonstrate that they are safe for human use (Baldrick, 2006). This lack of specific regulatory guidance led the International Pharmaceutical Excipients Council (IPEC), an industry association which champions excipients, to publish safety evaluation guidance for these materials (IPEC, 1997; Steinberg et al., 1996), while in Japan a paper relating to testing requirements of excipients has been released (Uchiyama, 1999). More recently, the United States (US) Food and Drug Administration (FDA) have issued guidance on nonclinical studies needed to develop excipients (FDA, 2005), with the key message that excipients are potential toxicants and need to be evaluated accordingly. Although no specific guideline exists in Europe, it has been stated that the toxicology and pharmacokinetics of an excipient used for the first time in the pharmaceutical field should be investigated (CPMP, 2000). Furthermore, when a new excipient is introduced on the market in Europe, it needs to have been evaluated by appropriate nonclinical studies and undergo a risk-benefit assessment (CHMP, 2007).

Testing strategies proposed by IPEC and the FDA offer a useful starting point for preclinical excipient testing. These strategies have been discussed elsewhere (Baldrick, 2006) but are summarised in Table 1. The extent of testing will need a case-by-case approach based on duration and route of use of the excipient and also whether development of the new material is for "stand alone" use for inclusion in a range of drug formulations, or as a material for use in a specific drug formulation only. In the latter case, testing requirements can be reduced as the excipient can be tested in studies with the drug itself, perhaps with an additional excipient-only test group. A further consideration that may reduce testing requirements is whether the material proposed for excipient use has had prior human use/exposure in food, cosmetics or medical devices, or from the chemical industry.

This paper will examine whether chitosan has the potential to be a safe pharmaceutical excipient based on publicly available data and aims to assist in limiting nonclinical testing needed to include the material in future drug formulations.

2. Chitosan

Chitosan is a polysaccharide comprising copolymers of glucosamine (β(1–4)-linked 2-amino-2-deoxy-D-glucose) and *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) and can be derived by partial deacetylation of chitin from crustacean shells (Illum, 1998; Jones and Mawhinney, 2006). Its discovery has been attributed to Rouget in 1859 when he found that boiling chitin in potassium hydroxide rendered the material soluble in organic acids; Hoppe-Seyler named it chitosan in 1894 (Khor, 2001). However, it took until 1950 for the structure of chitosan to be finally resolved. In the literature, the term chitosan is used to describe

E-mail address: paul.baldrick@covance.com

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doi:10.1016/j.yrtph.2009.09.015

Table 1
Preclinical testing strategies for new excipients.

Testing source	Recommended nonclinical study for each development stage			
	Initial clinical use	Short-term clinical use	Mid-term clinical use	Longer-term clinical use
IPEC-US[a,e]	–	Acute oral and dermal toxicity, skin and eye irritation and skin sensitisation. Bacterial gene mutation and chromosome damage. ADME (intended route). 28-day toxicity (2 species by intended clinical route)	Short term use studies. 90-day toxicity (most appropriate species). Teratology (rat and/or rabbit). Genotoxicity assays. Additional assays (conditional)[h]	Short/mid term studies. One generation reproduction. Chronic toxicity (rodent and nonrodent) and carcinogenicity (conditional)
IPEC-Europe[b,f]	ADME[d]	Acute toxicity (intended route) and skin sensitisation. Ames, chromosome damage and micronucleus. Four week toxicity (2 species by intended route)	Short term use studies. Three-month toxicity (most appropriate species). Teratology (rat and rabbit). Genotoxicity assays	Short/mid term studies. Segment I reproduction. 6–9 month toxicity (rodent and nonrodent), segment III reproduction and carcinogenicity (conditional)
FDA[c,g]	Standard safety pharmacology battery	Acute toxicity (rodent and nonrodent by intended route; although option of not performing these studies if sufficient dose levels are used in repeat dose studies). ADME (intended route). Standard genotoxicity battery. One-month toxicity (rodent and nonrodent by intended route). Single study rodent assay to evaluate all phases of reproductive toxicity and a teratology study in a nonrodent	Short term use studies (although option of not performing 1-month studies). Three-month toxicity (rodent and nonrodent species by appropriate route). Parenteral use studies (conditional)	Short/mid term studies (although option of not performing 1- and 3- month studies). Six-month toxicity in rodent and chronic toxicity in nonrodent (by appropriate route). Carcinogenicity in 2 rodent species or one rodent species plus, for example, a transgenic model (conditional)

[a] Steinberg et al. (1996).
[b] IPEC (1997).
[c] FDA (2005).
[d] Absorption, distribution, metabolism and excretion.
[e] Additional considerations for inhalation/intra-nasal route: acute inhalation, application site and pulmonary sensitisation studies; for parenteral route: acute parenteral toxicity and application site studies; mucosal use: application site evaluation; transdermal and topical drugs: application site and phototoxicity/photoallergy evaluation. Photocarcinogenicity is a conditional option for transdermal and topical excipients.
[f] Additional considerations for mucosal, transdermal, dermal/topical, parenteral, inhalation/intra-nasal and ocular use: skin and eye irritation and application site studies; for parenteral route: acute parenteral toxicity study. Pulmonary sensitisation is a conditional option for inhalation/intra-nasal excipients and phototoxicity/photoallergy plus photocarcinogenicity are conditional options for transdermal and dermal/topical materials.
[g] Additional considerations for topically (dermal, intra-nasal, intraoral, ophthalmic, rectal or vaginal) or pulmonary administered excipients are ocular irritation, sensitisation, oral or parenteral route toxicity studies; additional considerations for injectable excipients are an *in vitro* haemolysis study, measurement of creatinine kinase and protein binding evaluation; where appropriate new excipients should also be examined for photosafety.
[h] Studies specific to the nature of the excipient, for example, screening for endocrine modulators.

chitosan polymers with different molecular weights (50–2000 kDa), viscosity and degree of deacetylation (40–98%) (Illum, 1998). Although not strictly adhered to, it is reported that the generic term chitosan is applied when the extent of deacetylation is above 70% and the generic term chitin is used when the extent of deacetylation is insignificant, or below 20% (Pusateri et al., 2003). Chitosan is known by a number of other names including polyglusam, deacetylated chitin and poly-D-glucosamine. The major component of chitosan, glucosamine, is a natural substance produced in the body from glucose and is involved in the manufacture of glycosaminoglycan, which forms cartilage tissue in the body; glucosamine is also present in tendons and ligaments (Anderson et al., 2005; Martindale, 2007).

Interest in the use of chitosan as a pharmaceutical excipient is not new but it still does not appear to be present in any marketed drugs. However, it is reported as being under investigation for use in a number of pharmaceutical formulations including drug delivery applications, muco-adhesive dosage forms, rapid release forms, improved peptide delivery, colonic drug delivery systems and for gene delivery (Jones and Mawhinney, 2006). Potential use in tablets (including controlled release), as well as wet granulations, gels, films, emulsions, wetting agents, coating agents and microspheres and microcapsules has been explored (Illum, 1998). Possible use of chitosan as an oral nanoparticle drug carrier has also been reported (Morishita and Peppas, 2006; Wilson et al., 2009), as has use as in an intra-nasal chitosan-based vaccine (Illum et al., 2001; Read et al., 2005).

Although not as a pharmaceutical excipient, established human exposure to chitosan has occurred through its use as a dietary supplement in preparations for obesity and hypercholesterolaemia (Illum, 1998; Martindale, 2007). Indeed, chitosan-based supplements are sold as fat trappers and magnets (Gades and Stern, 2003). Various chitosan-containing medical devices such as the haemostatic Celox for the treatment of bleeding and bandages such as HemCon or QuikClot for the control of bleeding are marketed in Europe and the US (CDRH, 2006; Millner et al., 2009). Use for contact lens coatings or as contact lens material itself has also been explored (Illum, 1998). Other work has investigated the use of chitosan in wound healing (Kojima et al., 2004; Muzzarelli et al., 1999); the chitosan-containing wound healing product Tegasorb was marketed in the past (Illum, 1998) but appears now to have been withdrawn. It also has been shown to have potential utility for use in periodontal disease (Boynuegri et al., 2009; Ikinci et al., 2002). In the food industry, chitosan (ChitoClear product) has been designated as Generally Recognised As Safe (GRAS) in the US for use in foods in general (CSFAN, 2009). Furthermore, chitosan is listed as a food additive in Japan (Tago et al., 2007) as well as Italy and Finland (Illum, 1998). In the cosmetic industry, chitosan is used as a base material (Minami et al., 1996). Chitosan has industrial use as a flocculant and chelating agent, in the clarification of beverages and as a fungicide for crop protection (Illum, 1998).

3. Biological activity

Conventionally, pharmaceutical excipients have been viewed as "inert" materials although this view has changed as new proposed excipients cover a range of functions from stabilising formulations to active roles of enhanced drug uptake and specific drug delivery. However, their role is not to have "pharmacological" activity. Such a situation appears to be the case for chitosan although its medical device use and wound healing potential has centred around "biological" activity.

A range of studies have shown that chitosan acts as a haemostatic agent and some of these are summarised in Table 2. *In vitro* studies with blood have indicated that its haemostatic mechanism seems to be independent of the classical coagulation cascade and appears to be due to an interaction between the cell membrane of erythrocytes and chitosan, with clot formation in the absence of coagulation factors or platelets (Klokkevold et al., 1999; Rao and Sharma, 1997). It has been shown that chitosan, with its reactive amino groups, interacts with blood cells because it carries a positive charge (Mathews et al., 2006; Sagnella and Mai-Ngam, 2005). It is thought that, as the outer membranes of erythrocytes and platelets are negatively charged, they get attracted to the positively charged chitosan leading to platelet activation and thrombus formation. Furthermore, in the case of blood contacting applications such as drug delivery systems, it has been reported that the positively charged chitosan tends to attract circulating plasma proteins, which adsorb to the material surface, which in turn results in platelet adhesion, activation on the surface of the material and thrombus formation (Sagnella and Mai-Ngam, 2005). Such action has been utilised in medical device products such as Celox and chitosan-containing dressings for the treatment of bleeding and have been supported by evidence of reduced bleeding and enhanced haemostasis in experimental animal models. A role for chitosan in the wound healing process has been demonstrated through haemostatic action, macrophage activation and stimulation of cell proliferation and histo-architectural tissue organisation (e.g. Muzzarelli et al., 1999; Paul and Sharma, 2004) with supporting evidence from induced wound models.

The role of chitosan in the nutritional supplement market as a weight loss aid and cholesterol lowering agent arose from work in the rat showing cholesterol-lowering effects in rats given chitosan diets (Sugano et al., 1980). Such work and some related studies are summarised in Table 2. Although still not fully understood, chitosan's activity has been related to its positively charged nature resulting in binding to free fatty acids (released from consumed fat) and bile salt components which results in disrupted lipid absorption in the gut (Illum, 1998). It has been suggested that chitosan dissolves in the stomach, emulsifying fat and forming a gel in the intestine which entraps fat and prevents intestinal absorption (Kanauchi et al., 1995; Ylitalo et al., 2002). More recently, it has been proposed that chitosan forms a flocculus in the duodenum which entraps dietary oil (Rodriguez and Albertengo, 2005). However, whether chitosan is actually clinically effective in cholesterol control or weight loss remains controversial, with work indicating that chitosan dietary supplementation had no effect on fat absorption or plasma cholesterol levels and had only a minimal, clinically insignificant, effect on bodyweight (Gades and Stern, 2003; Mhurchu et al., 2005; Tapola et al., 2008). Other studies, however, have described reduced blood cholesterol and lower weight, percentage fat and fat mass in the clinic from chitosan supplementation (Kaats et al., 2006; Ylitalo et al., 2002).

A variety of other biological effects have been attributed to chitosan including anti-ulcerogenic, antimicrobial, anti-tumour, immune modification, renal protective and osteogenetic actions and use in tissue engineering as a bio-scaffold to allow skin or bone cell growth (e.g. Tago et al., 2007), and is supported by a range of *in vitro* and *in vivo* studies examining potential efficacy.

4. Pharmacokinetics

A number of studies have examined the *in vitro* and *in vivo* degradation of chitosan, often as films or from implantation, while its fate following oral administration is less well reported. It has been shown that the *in vitro* degradation of chitosan with egg-white and human lysozyme occurs mainly through depolymerisation by the enzyme (Varum et al., 1997). Degradation is dependent on the degree of deacetylation, being less rapid as it becomes higher

Table 2
Biological activity of chitosan.

Study[a]	Results	Reference
Effect of chitosan in lingual haemostasis model in rabbits	Enhanced haemostasis due to decreased bleeding time	Klokkevold et al. (1991)
Action of chitosan on blood from various species	Haemagglutination and clot formation demonstrated	Rao and Sharma (1997)
Effect of chitosan dressing on healing at split skin graft donor sites in human patients	Rapid wound healing demonstrated	Stone et al. (2000)
Tail bleeding and effect of application to full thickness skin incisions in mice with chitosan hydrogel	Rapid cessation of bleeding and accelerated wound healing	Ishihara et al. (2002)
Application of chitosan in a severe hepatic injury model in swine	Reduced blood loss and increased haemostasis and survival	Pusateri et al. (2003)
Implantation of fabric impregnated with chitosan into skin wounds of rats	Evidence of an early wound healing process	Kojima et al. (2004)
Effect of chitosan in a rat model of artificially induced peritoneal adhesion	Adhesion reduced	Zhang et al. (2006)
Application of chitosan-containing haemostatic agent Celox in a haemorrhagic groin injury model in pigs	Reduced bleeding and increased survival	Kozen et al. (2008)
Dietary administration of chitosan (for example, up to 5%) to rats, either with a normal or low/high cholesterol diet	Reduced blood and liver cholesterol, lowered cholesterol and oleic acid absorption and altered intestinal bile acid metabolism were demonstrated	Sugano et al. (1980), Vahouny et al. (1983), Jennings et al. (1988), Fukada et al. (1991)
Dietary feeding of chitosan to rats with a high cholesterol diet	Reduced liver cholesterol and cholesterol absorption and increased faecal fat and bile acid excretion were shown	Gallaher et al. (2000)
Dietary feeding of chitosan to mice	Reduced blood glucose, cholesterol and triglyceride	Miura et al. (1995)
Dietary feeding of chitosan to rabbits with a high cholesterol diet	A hypocholesterolaemic action in the intestine was shown by increased faecal bile acid and sterol levels	Hirano and Akiyama (1995)
Oral administration of chitosan to dogs	Decreased plasma cholesterol seen	Ylitalo et al. (2002)

[a] Only some of the studies referenced give robust information on the degree of acetylation and/or molecular weight of the chitosan material used, and as it is not considered to affect the overall interpretation of the presented data, has not been included.

(Tomihata and Ikada, 1997; Ren et al., 2005) and minimal degradation of films with very low or high acetylation (Freier et al., 2005). Subcutaneous implantation of chitosan films in rats (for up to 12 weeks) also showed that degradation occurred less rapidly as the degree of deacetylation became higher (Tomihata and Ikada, 1997). In discussing tissue implantation use, it is reported that highly deacetylated forms (>85%) show the lowest degradation rates and may last several months *in vivo*; material with lower levels of deacetylation degrades more rapidly (Hutmacher et al., 2001). The degradation products are chitosan oligosaccharides of variable length. Further work following subcutaneous implantation of a slow degrading chitosan hydrogel in rats showed no detectable gel fragments in distant organs (brain, heart, lungs, liver, spleen, kidney and sternal bone marrow) at sacrifice times ranging from 4 to 30 days (Azab et al., 2007). Degradation of the gel was suggested to be due to an oxidative process at the dose site. An examination of chitosan gel beads as a potential vehicle for drug delivery showed biodegradation from within 3 to between 14 and 28 days following subcutaneous implantation in mice; degradation was accelerated as the degree of deacetylation decreased (Kofuji et al., 2002). In the context of drug carrier resorption and wound healing, it is reported that chitooligomers and monomers are generated from chitosan biodegradation by lysozyme, *N*-acetylglucosaminidase and human chitinase (Muzzarelli, 1997). It has also been shown that serum lysozyme levels were elevated following intravenous dosing of rabbits with chitosan oligosaccharides (Hirano et al., 1991). Intraperitoneal injection of fluorescin isothiocyanate-labelled chitosan to mice resulted in rapid exposure to the kidney and urine, with scarce distribution to the liver, spleen, abdominal tissue and plasma (Onishi and Machida, 1999). Most of the labelled material was excreted into urine after 14 h, as a small molecular weight product. It was concluded that chitosan is highly biodegradable and easily excreted in urine, and showed no issues of accumulation/retention in the body.

Absorption of fluorescin isothiocyanate-labelled chitosans (with varying molecular weights and degrees of deacetylation) was investigated in Caco-2 cells and following oral administration to rats using 20 mg/kg (Chae et al., 2005). Results showed that as molecular weight increased, absorption decreased. Compared to high molecular weight chitosan (230 kDa), absorption increased by more than 20-fold compared with lower weight material (3.8 kDa) in both *in vitro* and *in vivo* assessment. Plasma C_{max} and AUC values ranged from <0.5 up to 20.23 μg/mL and 0.97 up to 24.13 μg mL h, respectively. Dense fluorescence intensities were seen in the epithelium of villi in duodenum and jejunum with the 3.8 kDa chitosan and intensities decreased with increasing chitosan molecular weight, with no sign of absorption observed with the 230 kDa form.

Although the bioavailability of chitosan after oral administration to animals has not been well investigated, substantial amounts have been shown to be digested in the gut of the rabbit (Hirano et al., 1990; Lee et al., 2004). Indeed, chitinolytic enzymes digesting chitosan to generate *N*-acetylglucosamine (a copolymer of chitosan) are present in the intestinal mucosa of animals as well as in intestinal bacteria. An investigation of intestinal absorption of ^{14}C-chitosan in rats showed that the material underwent digestion into low molecular weight substances within the gastrointestinal tract, and that it was distributed extensively in tissues (Nishimura et al., 2003). Examination of faeces showed that chitosan was reduced to 6–18% of its original weight, following oral dosing of nylon bags packed with the material in dogs; administration into the jejunum and colon showed about 40% loss (Okamoto et al., 2001). Interestingly, it has also been pointed out (although with no evidence provided) that chitosan is not specifically hydrolysed by digestive enzymes in man, but limited digestion due to bacterial flora and unspecific enzymes such as amylase and lipase might occur (Ylitalo et al., 2002).

Oral administration of ^{14}C-labeled *N*-acetylglucosamine to rats resulted in excretion in the urine, faeces and expired air of 4%, 17% and 54%, respectively, during 1 week after dosing; the other 25% was distributed into connective tissue or cartilage (Lee et al., 2004). Oral and intravenous studies in rats and dogs with radiolabelled glucosamine (another copolymer of chitosan) showed rapid clearance by the liver and kidney and excretion in the

urine for a part of the dose, with the remainder ending up in skeletal muscle and articular cartilage (Anderson et al., 2005). The chitooligomers, which are produced by depolymerisation of chitosan, have been reported to have good intestinal absorption (Qin et al., 2006).

5. Toxicology and related safety testing

A range of toxicology studies have been performed with chitosan and related materials as well as work examining for biocompatibility and implantation effects for medical device use.

Table 3
General toxicology data for chitosan and related materials.

Study[a]	Results	Reference
Single dose various species		
LD_{50} value of chitosan	Oral rat: >1500 mg/kg Intraperitoneal rat: 3000 mg/kg Subcutaneous mouse: >10,000 mg/kg Intraperitoneal mouse: 5200 mg/kg	Minami et al. (1996)
	Oral mouse: >16,000 mg/kg	Jones and Mawhinney (2006)
LD_{50} value of chitooligomers (produced by depolymerisation of chitosan)	Oral mouse: >10,000 mg/kg (no deaths or clinical signs of toxicity in dose range of 1000–10,000 mg/kg)	Qin et al. (2006)
Repeat dose rat		
Dietary administration of *N*-acetylglucosamine (a copolymer of chitosan) to groups of 10 male and 10 female rats at 0%, 0.625%, 1.25%, 2.5% or 5% (corresponding to 0, 302–351, 588–695, 1218–1412 or 2476–2834 mg/kg/day) for 13 weeks	No obvious toxicity seen (among males, there was a increase in bodyweights along with daily food intake, perhaps due to the sweet flavour of *N*-acetylglucosamine)	Lee et al. (2004)
Dietary administration with glucosamine (another copolymer of chitosan) in rats at 300–2700 mg/kg/day for 52 weeks and dogs at 159–2149 mg/kg/day for 26 weeks	No adverse findings reported	Anderson et al. (2005)
Dietary administration of chitooligomers (produced by depolymerisation of chitosan) at 0%, 0.75%, 1.5% or 3% (corresponding to levels of 0, 750, 1500 or 3000 mg/kg/day) to in groups of 10 male and 10 female rats over 30 days	No study findings seen	Qin et al. (2006)
Oral gavage administration of either 0, 500, 1000 or 2000 mg/kg/day chitosan oligosaccharide (also known as oligoglucosamine and prepared by hydrolysis of chitosan) to groups of 9 male and 9 female rats for 4 weeks or to groups of 10 male and 10 female rats using 0%, 0.04%, 0.2% or 1% (corresponding to 0, 25–27, 124–142 or 653–720 mg/kg/day) in the diet over 90 days	Oral gavage dosing showed no study findings. Dietary administration had findings confined to the high dose level and included erythema and swelling of the snout and forelimbs and, loss of fur on the forelimbs along with macroscopic emaciation, swelling of the snout, auricles and forelimbs and, alopecia of the forelimbs. Other findings were decreased bodyweight gain and food consumption, increased platelet count, lymphocyte count and differential neutrophil count abnormalities in urinalysis (including proteinuria) and blood chemistry (including decreased blood protein, creatinine, glucose, total cholesterol and triglyceride), as well as a small thymus, small spleen, dark spots or areas on the glandular stomach mucosa, pale Harderian glands and small testes along with vacuolised Sertolic cells and decreased germ cells. A dietary level of 0.2% produced no signs of toxicity	Kim et al. (2001), Naito et al. (2007)
Dietary administration of oligo-*N*-acetylglucosamine (consists of the monomer and oligomers of *N*-acetyl-D-glucosamine) to groups of 10 male and 10 female rats at 0.2%, 1% or 5% for 90 days (with the latter 2 dose levels reported as corresponding to 641 or 3640 mg/kg/day)	No obvious toxicity was seen and study findings were limited to occasional increases in food consumption (perhaps due to the sweet nature of oligo-*N*-acetylglucosamine in the diet) in males at the high dose level	Tago et al. (2007)
Dietary administration of *N*-acetylglucosamine to groups of 10 male and 10 female rats at 0%, 1.25%, 2.5% or 5% (corresponding to 0, 580–647, 1159–1269 and 2323–2545 mg/kg/day) for 52 weeks	No obvious toxicity (a slight suppression of bodyweight gain at the higher dose levels was attributed to slight reduction of caloric intake due to high concentration of test material)	Takahashi et al. (2009)
Repeat dose mouse, rabbit and dog		
Intraperitoneal and subcutaneous dosing of 5 mg chitosan to mice every 2 weeks over a 12-week period and dietary administration of up to 5% over 4 weeks	Intraperitoneal dosing resulted in decreased bodyweights along with clinical signs of emaciation, pilomotor, depilation around the rostrum and eyelids, oedema of the auricle, rostrum and eyelids and photophobia and, histological changes of macrophages with hyperplasia in the mesenterium. Following subcutaneous injection, the dose site was swollen and contained polymorphonuclear cells and cell debris. Dietary administration at 5% resulted in decreased bodyweight as well as a reduction in the number of *Bifidobacterium* and *Lactobacillus* in normal flora of the intestinal tract, although there was no effect with a 0.5% diet	Tanaka et al. (1997)
Repeat dosing of chitosan (10 days with a solution formulation and 14 days with a powder formulation) to rabbits (no further details are given)	No macroscopic or histopathological changes in organs and tissues (no further details are given)	Illum (1998)
Intravenous injection of chitosan to rabbits at 4.5 and 50 mg/kg/day for 10 days and oral dosing at 700–800 mg/kg/day for 34 weeks	Intravenous injection at 4.5 mg/kg/day produced no abnormal changes, while 50 mg/kg/day caused death. Oral dosing produced no evidence of toxicity	Hirano et al. (1990), Carreno-Gomez and Duncan (1997)
Subcutaneous dosing of chitosan to mongrel dogs for 1 month at 0, 10, 30, 50, 100, 150 and 200 mg/kg/day	Vigour loss and anorexia were seen from 50 mg/kg/day (transient at 30 mg/kg/day), with deaths from 150 mg/kg/day and evidence of severe dyspnoea. Increased white blood cell count (due to neutrophilia) was seen from 50 mg/kg/day, while raised lactate dehydrogenase and creatine kinase (notably at 200 mg/kg/day) occurred. Severe pneumonia was noted in dogs that died	Minami et al. (1996)

[a] See footnote for Table 2.

5.1. General toxicity

The single dose toxicity of chitosan in rodents is low with reported values given in Table 3. This table also summarises repeat dose toxicity studies that have been performed with chitosan or related materials in rodents, rabbits and dogs using various parenteral and oral (gavage and dietary) routes. The dietary studies appear to have largely been performed to support the safe use of chitosan materials for food use. In general, results showed no toxicity in rats at up to 2000 mg/kg/day from gavage dosing and at up to 5% (approximately 3000 mg/kg/day) in the diet for durations of up to 3 months. Apparent toxicity was seen at a dietary level of 1% (corresponding to 653–720 mg/kg/day) chitosan oligosaccharide (Naito et al., 2007). However, it has been suggested that topical findings including erythema/hair loss and swelling of the snout and forelimbs might be due to dermal responses to chitosan oligosaccharide adhering to the skin and fur, which are easily soiled with saliva during grooming, while decreased bodyweight gain and food consumption may be related to feeding difficulties due to the topical lesions on the snout and forelimbs. It is further suggested that increased platelet count, lymphocyte count and differential neutrophil count may be related to the dermal inflammation, although it is not clear if abnormalities in urinalysis and blood chemistry, as well as various organ weight and macroscopic pathology findings, along with vacuolised Sertolic cells and decreased germ cells were related only to malnutrition. The overall toxicological significance of these findings is unclear as a dietary level at 0.2% (corresponding to 124–142 mg/kg/day) chitosan oligosaccharide produced no signs of toxicity, as did oral gavage dosing of the same material at up to 2000 mg/kg/day (Kim et al., 2001).

A number of other studies examining the effects of feeding chitosan diets to rats (which would have resulted in high g/kg levels) have shown that it is generally well tolerated. Many of these studies were performed to examine effects on lipid levels to support a role for chitosan to treat clinical obesity and hypercholesterolaemia and only a few examples from the literature are mentioned here. Early experiments in which 2%, 5% and 10% chitosan was given in a high cholesterol diet only showed depressed growth and food intake at the high dose levels; plasma cholesterol was lowered in all groups compared to controls and liver weight decreased with increasing amounts of chitosan (Sugano et al., 1980). Feeding of 1% and 5% chitosan diets for 4 weeks showed no effect on bodyweight compared to controls; oleic acid and cholesterol absorption was lowered in the high dose group (Vahouny et al., 1983). No significant effect on growth, food intake or liver weight was seen in rats fed 2% or 5% chitosan in a low cholesterol diet for 3 weeks; the high dose level reduced cholesterol levels and evidence of altered intestinal bile acid metabolism was seen (Fukada et al., 1991). Histopathological examination of the small and large bowel following feeding of 5% chitosan to rats showed no changes (Jennings et al., 1988). In a study to examine for potential effects from chitosan present in animal feeds containing by-products recovered from food processing wastes by coagulation with chitosan, diets of up to 5% chitosan over 8 weeks in rats showed no adverse findings (Landes and Bough, 1976). Progressive growth reduction and various clinical pathology disturbances were seen only at high levels of 10% and 15% dietary chitosan, along with enlargement of the liver and kidneys at the highest dose level.

Findings in mice following parenteral dosing appear to be due to the high doses used (5 mg chitosan, which would have given a high mg/kg level) with macrophages and hyperplasia in the mesenterium following intraperitoneal dosing and dose site pathology following subcutaneous injection (Tanaka et al., 1997). These histological changes were considered to be associated with the slow clearance of a large amount of injected material. Further work in mice showed that dietary levels of up to 5% were well tolerated. High dose effects were also seen in rabbits following intravenous dosing of chitosan, with deaths at 50 mg/kg/day (but no effects at 4.5 mg/kg/day) (Carreno-Gomez and Duncan, 1997). It was suggested that the finding was probably due to blood cell aggregation. However, oral dosing at up to 800 mg/kg/day in this species was well tolerated. In work examining cholesterol levels in dogs, oral dosing in the range of 1000–6000 mg over a 2-week period was well tolerated, with only a slight reduction in bodyweight (and lowered plasma cholesterol) at the high dose level (Ylitalo et al., 2002). Other work in dogs showed evidence of toxicity following subcutaneous dosing with clinical signs from 30 mg/kg/day, clinical chemistry changes (especially neutrophilia) from 50 mg/kg/day and severe dyspnoea and deaths at 150–200 mg/kg/day (Minami et al., 1996). Pathological examination showed severe pneumonia in the latter animals and it was suggested that this finding was possibly induced by immunological reactions and cytokine activation. Indeed, further work in dogs showed that subcutaneous levels as low as 10 mg/kg chitosan activated complement; subcutaneous dosing of mice with 50 mg/kg chitosan also activated complement (Minami et al., 1998).

5.2. Genotoxicity

Literature data reporting modern genotoxicity testing for chitosan itself are limited. Chitooligomers (produced by depolymerisation of chitosan) showed no evidence of mutagenicity in a reverse mutation bacterial assay using *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 at up to 5000 μg/plate, with or without metabolic acid (Qin et al., 2006). In a bone marrow micronucleus test, following oral gavage dosing of the material to mice at up to 5000 mg/kg on two occasions, 24 h apart, no evidence of micronucleus induction was seen. Furthermore, oral gavage dosing of chitooligomers to male mice at up to 5000 mg/kg for 5 days showed no morphological changes in sperm. Although study details are not given, neither photocrosslinkable chitosan or its chitosan hydrogel were reported to give positive responses in mutagenicity testing (Ishihara et al., 2002). Finally, antigenotoxic activities have been reported for chitosan in the sister chromatid exchange assay using Chinese hamster lung cells (Ohe, 1996). The material reduced the marked increase in sister chromatid exchanges induced by the mutagens 4-nitroquinoline-*N*-oxide, dinitropyrene, mytomycin C and adriamycin. Antimutagenic activity has also been reported for chitosan oligosaccharides when tested in reverse mutation bacterial, Umu gene expression and *Bacillus subtilis* Rec assays (Nam et al., 2001).

Data for the copolymers of chitosan are also available. In a reverse mutation bacterial assay using *S. typhimurium* strains TA98 and TA100, *N*-acetylglucosamine showed no evidence of mutagenicity (Lee et al., 2004). Furthermore, although some possible positive findings were seen in non-standard assays, glucosamine (as the hydrochloride salt) was shown to be non-genotoxic when tested in a reverse mutation bacterial assay using *S. typhimurium* strains TA98, TA100, TA1537 and TA1537 and *Escherichia coli* strain *WP2uvrA* and in a mouse bone marrow micronucleus study (Anderson et al., 2005).

5.3. Reproduction toxicology

No data on the reprotoxic potential of chitosan or related materials was found in the public literature.

5.4. Carcinogenicity

No data on the carcinogenic potential of chitosan was found in the public literature. The chitosan constituent *N*-acetylglucosamine showed no evidence of carcinogenicity when dosed at levels

Table 4
Other safety data for chitosan and related materials.

Study[a]	Results	Reference
Cytotoxicity and haemocompatibility		
In vitro blood compatibility testing using human blood with chitosan	Coagulation, thrombus formation and platelet adhesion properties demonstrated	Lee et al. (1995)
In vitro biocompatibility of chitosan polymers and chitosan microspheres towards murine melanoma B16F10 cells and the ability to lyse rat erythrocytes	Cytotoxicity was demonstrated, with an inhibitory concentration $(IC)_{50}$ of 0.21 mg/mL for chitosan hydrochloride and release of haemoglobin occurred in the lysis assay in the 1 μm/mL–3 mg/mL range. Scanning electron microscopy showed damage to the erythrocyte membrane, cell aggregation and complete lysis	Carreno-Gomez and Duncan (1997)
Testing of photocrosslinkable chitosan and its chitosan hydrogel in cell culture tests of human fibroblasts, endothelial cells or smooth muscle cells	No cytotoxicity seen	Ishihara et al. (2002)
Haemocompatibility in canine blood with chitosan	Reduced blood coagulation time, increased platelet aggregation and cytokine (platelet derived growth factor-AB and transforming growth factor-β1) release from platelets were demonstrated	Okamoto et al. (2003)
In vitro cytotoxic effects of various chitosans in Caco-2 cells	Little or no cytotoxicity was seen at low concentration (1 mg/mL) while effective concentration $(EC)_{50}$ values for cell viability in the range of 3.2 to >20 mg/mL were seen at higher concentrations	Chae et al. (2005)
In vitro cytotoxicity testing of chitosan salts with Caco-2 cells	Cytotoxicity was demonstrated with IC_{50} values in the range of 0.22–0.72 mg/mL	Opanasopit et al. (2007)
Haemocompatibility measured from haemoglobin released from blood by haemolysis and cytotoxicity to mouse fibroblasts from chitosan membranes	A non-haemolytic result and a low level of cytotoxicity were demonstrated	Mohy Eldin et al. (2008)
Local tolerance and implantation		
Skin irritation testing in guinea pigs plus eye irritation and pyrogenicity testing in rabbits with chitosan extract (dose level not specified)	No findings were reported	Rao and Sharma (1997)
Intra-nasal administration (50 μL/nostril) of 0.25% formulations of chitosan to guinea pigs for 28 days	No macroscopic abnormality to the nasal tissue was seen	Aspden et al. (1997)
Single dose intra-nasal administration of 0.125%, 0.25% and 1% chitosan hydrochloride to rats	No effects on rat nasal epithelia occurred at lower concentrations; a slight change in the shape of the nuclei was seen at 1%	Haffejee et al. (2001)
Ocular administration of (a) chitosan gels (4 instillations a day for 3 days) or (b) chitosan-coated nanoparticles (2 instillations per hour for 6 h) to rabbits (dose levels not specified)	Results showed (a) evidence of low irritation and (b) no irritation or appreciable disruptions in the epithelial cells	(a) Alonso and Sanchez (2003) and (b) Calvo et al. (1997)
Intramuscular implantation of chitosan films in rabbits (dose level not specified) for up to 7 days	No adverse findings were seen	Rao and Sharma (1997)
Subcutaneous implantation of chitosan films in rats for a 12 week evaluation period	No significant foreign body reaction was demonstrated	Tomihata and Ikada (1997)
Implantation of chitosan scaffolds in mice with sacrifice at up to 12 weeks later	Only minimal signs of an inflammatory reaction reported	VandeVord et al. (2002)
(a) Subcutaneous implantation or intraperitoneal administration of 2 biodegradable chitosan hydrogels (fast degrading and slow degrading) to rats at 1000 mg/kg, with groups of 4 animals sacrificed on days 0, 1, 3, 7 and 14 (fast degrading gel) or days 0, 3, 7, 14 and 28 (slow degrading gel). (b) Further subcutaneous implantation of 0, 1000, 5000 or 15,000 mg/kg of slow degrading gel to rats, with sacrifice of groups of 5 animals on day 4, 14 and 30	(a) The adjacent tissue response to the gels after implantation was of a typical foreign body reaction (with no haemorrhage or necrosis). Signs of partial and total degradation were seen for the fast degrading gel at 7 and 14 days, respectively, after intraperitoneal implantation. (b) No tissue damage was demonstrated	Azab et al. (2007)

[a] See footnote for Table 2.

of up to 5% in the diet (corresponding to 1935–2244 mg/kg/day) of rats for 2 years (Takahashi et al., 2009).

5.5. Cytotoxicity and haemocompatibility

A number of studies have examined the cytotoxicity and haemocompatibility of chitosan and some examples are given in Table 4. In agreement with its biological activity as a haemostatic agent described earlier, *in vitro* studies with blood showed evidence of coagulation, thrombus formation and platelet adhesion/aggregation. Other work in cell cultures has shown that chitosan polymers can be cytotoxic at concentrations in the μm/mL to mg/mL range, with the level of effect dependent on the salt form used and polymer molecular weight (Carreno-Gomez and Duncan, 1997; Chae et al., 2005; Opanasopit et al., 2007); other workers, however, failed to shown cytotoxicity in various cell cultures (Ishihara et al., 2002). Lysis of erythrocytes by chitosan was also demonstrated. It has been pointed out that high molecular weight chitosans have the potential to induce cellular toxicity when used as polymeric carriers for intravenous administration (Carreno-Gomez and Duncan, 1997). In addition, it has been suggested that different binding affinity of the chitosans on cellular membranes results in different degrees of cytotoxicity (Chae et al., 2005).

5.6. Local tolerance and implantation

Examples of studies examining effects of local administration of chitosan are shown in Table 4. The material was shown have no obvious adverse local tolerance properties. Implantation studies in rodents report an expected and typical foreign body reaction due to the presence of the implanted material.

5.7. Immunotoxicity

No findings associated with immunotoxicity were reported from oral toxicity studies in rodents with chitosan materials. However as mentioned earlier, it has been suggested that pneumonia following subcutaneous dosing in the dog at high dose levels was possibly induced by immunological reactions and cytokine activation and chitosan was shown to activate complement from a level

of 10 mg/kg (Minami et al., 1996, 1998). It was also shown that feeding of 1 or 3 mg of chitosan in the diet of rats resulted in enhanced cytokine release in gut mucosa and spleen (Porporatto et al., 2005). Further work has shown that chitosan amino groups are recognised by the immune system, with examples including activation of macrophages and cytokine stimulation in mice, and adjuvant activity by inducing cell-mediated immunity and circulating antibody formation in mice and guinea pigs by chitosan materials (Nishimura et al., 1984, 1986; Shibata et al., 1997; Tokura et al., 1999). These findings provide useful support for a local role for chitosan in wound healing through macrophage stimulation (Kojima et al., 2004; Muzzarelli et al., 1999).

5.8. Human data

Generally limited data are available for use of chitosan as a dietary supplement. Levels of 4.5 g chitosan daily have been orally taken in human volunteers with no adverse effects reported (Gades and Stern, 2003). Even higher oral levels of up to 6.75 g were reported as safe (Tapola et al., 2008). It has been reported that short-term human trials of up to 12 weeks have shown no clinically significant symptoms including no evidence of an allergic response, although a low incidence of mild and transitory nausea and constipation was seen (Ylitalo et al., 2002).

6. Discussion

To date, despite a lot of reported interest as a potential pharmaceutical excipient, chitosan, a polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine, does not yet appear to be present as a formulation constituent in any marketed drugs. Special interest has been reported for absorption enhancement in drug formulations via oral, intra-nasal and ocular routes. As indicated by FDA guidance and IPEC recommendation (see Table 1), a range of safety testing is required to demonstrate that a novel excipient is safe, whether administration is by oral intake or by other dose routes in the clinic. A substantial body of such testing is already available for chitosan.

Although not a requirement for an excipient, a range of studies in animal models with chitosan materials have shown that it has biological activity in the form of haemostatic action. The exact mechanism of this action has not been clearly established but chitosan seems to work through a mechanism independent of the classical coagulation cascade that appears to involve an interaction between the cell membrane of erythrocytes (negative charge) and chitosan (positive charge), leading to involvement of platelets and rapid thrombus formation. An action in altering lipid absorption in the intestine and reducing blood cholesterol levels has also been shown for chitosan.

The kinetics of chitosan have been examined. Oral administration of radio-labelled chitosan to rats indicated digestion into low molecular weight substances in the gastrointestinal tract and systemic exposure to labelled material. Reports on the extent of digestion vary with high levels reported in the gut of rabbit (from chitinolytic enzymes present in the intestinal mucosa) and gastrointestinal tract of dogs, while it has been noted (albeit with no supporting data) that chitosan is not specifically hydrolysed by digestive enzymes in man, with only limited digestion occurring. Systemically available chitosan is likely to be degraded to constituent glucosamine and *N*-acetylglucosamine by enzymes such as lysozyme. This enzyme is reported as existing in various human body fluids and tissues, with concentrations from 4 to 13 mg/L in serum (Ren et al., 2005) and was shown to be elevated in rabbits following intravenous injection of a chitosan material. Furthermore, it has been reported that chitooligomers and monomers are generated from chitosan biodegradation by lysozyme, *N*-acetylglucosaminidase and human chitinase. Animal studies have shown that glucosamine undergoes rapid clearance by the liver and kidney and excretion in the urine for a part of the dose, with the remainder ending up in skeletal muscle and articular cartilage, while *N*-acetylglucosamine also undergoes excretion or is distributed into connective tissue or cartilage.

Acute and repeat oral dose toxicity studies with chitosan and related materials (including chitosan's copolymers glucosamine and *N*-acetylglucosamine) have shown that gram levels/kg are non-toxic (with no obvious target organ effects) although it needs to be borne in mind that the actual level of systemic exposure was not measured; chitosans have also shown no evidence of genotoxicity. However, higher dose findings have occurred following parenteral dosing in various species. Chitosan appears not to cause local irritation and implantation data showed either no significant dose site reactions or mild, typical foreign body reactions. Various *in vitro* biocompatibility studies have shown that chitosan materials have the ability to cause cytotoxicity and to lyse erythrocytes, although other work showed no cytotoxicity to various cells types. Due to its biological activity, haemocompatibility work with chitosan has demonstrated coagulation, thrombus formation and platelet adhesion/aggregation. No reproduction and limited carcinogenicity data appear to be available for chitosan materials.

Human exposure of gram levels of chitosan have occurred from oral dietary supplementation intake and no adverse findings appear to have occurred. An observed safe level of oral intake for the chitosan constituent glucosamine, which is used in health supplements, of up to 2000 mg/day has been established for human use (Hathcock and Shao, 2007).

So could chitosan be used as a safe pharmaceutical excipient? For oral use, the answer would appear to be yes as available toxicology data indicate that high oral doses in rodents and rabbits are generally well tolerated (albeit without knowledge of what was actually absorbed systemically). Furthermore, any chitosan that enters the body by absorption is not likely to cause any issue of accumulation/retention in the body, due to conversion to naturally occurring glucosamine derivatives which are either excreted or used in the amino sugar pool. How much more nonclinical testing would be needed to support its use in oral drug formulations as highlighted in Table 1? Fortunately, the answer to this question is very case-by-case but the available animal data as well as previous history of chitosan use as a safe human dietary supplement and use in the food industry would support limited further testing with chitosan alone. Thus, toxicity evaluation could be a part of necessary testing of the new drug itself as a formulation constituent, perhaps with a chitosan only dose level and would cover dosing in both a rodent and nonrodent. The same situation applies to reproduction toxicology work and, if performed with the new drug, carcinogenicity testing. Aside from impurity contamination during its manufacture, chitosan itself would not be expected to be genotoxic. Although limited, available data for chitosan materials showed this to be the case. However, to counter the impurity question, some genotoxicity testing is likely to be needed on the chitosan used. A further consideration would be sensitisation testing to evaluate for potential adverse effects in patients with crustacean allergy. All these principles could also apply to support other non-parenteral clinical dose routes such as inhalation, intra-nasal, ocular or skin use, obviously with more emphasis placed on potential local effects. Available published data indicate chitosan has no obvious adverse local tolerance properties.

The available data do not give a clear answer on the safe use of chitosan as a parenteral excipient. Its use as a medical device in the control of bleeding relies on the haemostatic biological nature of the material and studies have demonstrated local findings such as blood coagulation, thrombus formation and platelet adhesion/

aggregation. Although not consistently seen, *in vitro* studies have also demonstrated cytotoxicity findings. Thus, there is a potential concern for such effects when used in a blood contacting situation, especially by the intravenous route. Although oral toxicity studies have shown no specific effects on haematology, as mentioned earlier it is not known how much chitosan was actually available systemically. Limited published data showed that while intravenous levels of 4.5 mg/kg/day in rabbits showed no effects, a higher dose of 50 mg/kg/day likely caused blood cell aggregation. Also, although it is not clear if related to the biological nature of chitosan interacting with blood, subcutaneous dosing in the dog showed neutrophilia from 50 mg/kg/day and activated complement from 10 mg/kg. Studies associated with wound healing have also demonstrated local macrophage activation. Whether such findings would have a clinical implication from use of low levels of chitosan in parenteral drug formulations is unknown but it has been pointed out that the haemostatic mechanism of chitosan seems to be independent of the classical coagulation cascade and likely related to an interaction between the cell membrane of erythrocytes and chitosan itself (Klokkevold et al., 1999; Rao and Sharma, 1997). Furthermore, although chitosan treatment enhanced haemostasis by causing decreasing lingual bleeding time in a rabbit model, ear bleeding showed no effect on systemic bleeding time or systemic coagulation time (Klokkevold et al., 1991). Overall, as for its use as an oral excipient, toxicity evaluation of chitosan for parenteral use could be a part of necessary testing of the new drug itself as a formulation constituent, perhaps with a chitosan only dose level. However, a fuller examination of potential effects on blood clotting would likely need further testing.

In conclusion, chitosan has the potential to be a safe pharmaceutical excipient for non-parenteral, non-blood contact, use as shown by publicly available data. Further studies are needed to demonstrate such safe use by the parenteral route due to chitosan's biological haemostatic nature.

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